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Intraparenchymal NGF Infusions Rescue Degenerating Cholinergic Neurons

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Nerve growth factor (NGF) exerts both trophic (cell survival) and tropic (axonal growth-promoting) effects on several neuronal populations. In particular, its robust ability to prevent lesion-induced and spontaneous age-related basal forebrain cholinergic neuronal degeneration, and to promote mnemonic recovery, has suggested its potential use as a therapeutic agent in Alzheimer's disease. When infused intracerebroventricularly, however, NGF is associated with several adverse effects that make this delivery route impractical. The present study examined whether intraparenchymal infusions of NGF adjacent to cholinergic neuronal soma are an effective and well-tolerated means of providing NGF to degenerating cholinergic neurons. Cholinergic neuronal rescue together with axonal sprouting responses and local tissue damage in the brain were assessed in adult rats that underwent complete unilateral fornix transections, followed by intraparenchymal infusions of recombinant human NGF for a 2-week period. Intraparenchymal NGF infusions prevented the degeneration of 94.7 ± 6.6% of basal forebrain cholinergic neurons compared to 21.7 ± 2.6% in vehicle-infused animals ($p < 0.0001$). Cholinergic axons sprouted toward the intraparenchymal NGF source in an apparent gradient-dependent manner. Glial responses to intraparenchymal infusions were minimal, and no apparent toxic effects of the infusions were observed. Thus, when infused intraparenchymally, NGF rescues basal forebrain cholinergic neurons, alters the topography of axonal sprouting responses, and does not induce adverse affects over a 2-week infusion period. Intraparenchymal NGF delivery merits further study at longer term time points as a means of treating the cholinergic component of neuronal loss in Alzheimer's disease.

Key words: Nerve growth factor (NGF); Cholinergic system; Memory; Neurotrophic factors: Alzheimer's disease; Aging; Drug delivery; Regeneration

INTRODUCTION

Nerve growth factor (NGF) is the best characterized neurotrophic factor (15,29,30). During development of the nervous system, NGF supports the survival of sensory and sympathetic neurons, and influences basal forebrain cholinergic innervation of the hippocampus (15, 19,35). NGF continues to influence neuronal populations during adulthood: when infused intracerebroventricularly (ICV), NGF rescues adult cholinergic neurons from lesion-induced and spontaneous age-related degeneration (6–8,13,27,33), and promotes mnemonic recovery (3,6,16,17,31).

These findings have led to suggestions that NGF treatment could prevent or reduce cholinergic neuronal loss in the most common neurodegenerative disorder, Alzheimer's disease (1,23,30,39). However, intraventricular NGF infusions cause weight loss (hypophagia) (36), sprouting of sympathetic axons surrounding the cerebral vasculature (18,25), sprouting of primary sensory axons into the CNS (38), and migration of Schwann cells in the subpial space surrounding the brain stem and spinal cord (38). These noncholinergic effects of ICV NGF infusions render this treatment method impractical in chronic neurological disorders such as Alzheimer's disease. Clearly, a delivery method is required to circumvent limitations in the diffusion of large molecules such as NGF across the blood–brain barrier; in addition, accurately targeted and regionally restricted NGF delivery is required to influence cholinergic neurons without eliciting responses from noncholinergic neurons.

The present study was designed to examine whether delivery of NGF to a single intraparenchymal brain infusion site adjacent to cholinergic somata in the basal forebrain could elicit biological responsiveness without inducing intolerable adverse local effects after cholinergic lesions. Embedded with this design were the following questions: 1) does NGF diffuse for a sufficient distance to rescue degenerating neurons over the full rostral-to-caudal extent of the medial septal nucleus; 2) could NGF

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delivery to the region of the cholinergic neuronal soma rather than to neuronal axon terminals mediate neuronal rescue; and 3) does the intraparenchymal infusion elicit substantial glial reactivity or parenchymal damage after short-term application that might render this delivery method impractical for long-term therapeutic use? To address these questions, adult rats underwent unilateral fimbria-fornix lesions followed by intraseptal infusions of recombinant human NGF. Two weeks later, rats were sacrificed and brains were examined for cholinergic neuronal labeling and host inflammatory responses.

MATERIALS AND METHOD

Seventeen adult Fischer 344 rats weighing 150–165 g were experimental subjects. Animals had free access to food and water throughout the experimental period, and institutional, AALAC, and Society for Neuroscience guidelines with respect to animal care were strictly adhered to.

For all surgical procedures, animals were anesthetized deeply (0.35 ml of a combination of ketamine 50 mg/kg, rompun 2.6 mg/kg, and acepromazine 0.5 mg/kg). Complete unilateral lesions of the right fimbria-fornix were performed using a microscopically guided aspiration pipette, as previously described (26). In the same session, animals received implantations of intraparenchymal infusion devices at coordinates A/P 0.8 mm, M/L 0.5 mm, V/D 7.0 mm relative to bregma (22). Seven animals received continuous infusions of 0.71 μg/day of recombinant human NGF (100 μg/ml, generously supplied by Genentech, Inc.) in artificial CSF, and 10 animals received artificial CSF alone, at a rate of 0.30 μl/h for a 2-week period using Alzet 2002 miniosmotic pumps (Alza Corp., Palo Alto, CA) that were half-coated with paraffin.

Two weeks later, animals were reweighed and then sacrificed. Pumps were removed and remaining volumes of solutions within pumps were measured to ensure that pumps had emptied at the expected rate during the 2-week study period. Animals were transcardially perfused with 4% paraformaldehyde, and brains were removed, postfixed for 4 h in paraformaldehyde, then placed into 30% phosphate-buffered sucrose solution for 48 h. Brains were sectioned in the coronal plane at 40-μm intervals and examined for Nissl staining, low-affinity neurotrophin (p75) receptor immunocytochemistry (monoclonal IgG-192 antibody at 1:100 dilution, gift of Dr. C. E. Chandler), choline acetyltransferase (ChAT) immunocytochemistry (polyclonal rabbit antibody at 1:5000 dilution, gift of Dr. L. G. Hersh), and glial fibrillary acidic protein (GFAP) (monoclonal antibody from Boehringer Mannheim at 1:250 dilution). All immunocytochemical labeling was performed by 1) incubating free-floating sections for 24 h in primary antibody solution in 0.1 M Tris-saline containing 1% blocking serum and 0.25% Triton X-100; 2) incubation for 1 h with biotinylated goat anti-rabbit IgG (for polyclonal antibodies) or biotinylated horse anti-mouse IgG (for monoclonal antibodies; Vector Laboratories) diluted 1:200 with Tris-saline containing 1% blocking serum; 3) 1-h incubation with avidin-biotinylated peroxidase complex (Vector Elite Kit) diluted 1:1000 with Tris-saline containing 1% blocking serum; and 4) treatment for 3–15 min with 0.05% solution of 3,3′-diaminobenzidine, 0.01% H_2O_2, and 0.04% nickel chloride in 0.1 M Tris-buffer. Immunolabeled tissue sections were mounted onto gelatin-coated glass slides, air dried, and covered with Permount and glass coverslips.

Basal forebrain cholinergic neuron numbers were quantified through every sixth section of the medial septal region immunocytochemically labeled for ChAT, as previously described (31). Three septal sections were counted per animal at distances of 420, 500, and 640 μm rostral to the decussation of the anterior commissure, as previously described. Numbers of ChAT-labeled neurons per side of the brain were summed and divided into the number of neurons on the unlesioned side of the brain to obtain an average percentage of remaining neurons immunolabeled for ChAT per animal. Stereological quantification methods were not utilized because the purpose of the present study was to determine *relative* proportions of labeled cholinergic neurons in NGF- and vehicle-treated groups.

Glial responses to the intraparenchymal infusions were qualitatively evaluated on GFAP-labeled sections sampled at intervals of 240 um. General host cellular inflammatory responses were examined on Nissl-stained sections spaced 240 μm apart through the septal region, using morphological criteria to identify cells. Medium-sized, multinuclear cells with moderate amounts of cytoplasm were identified as polymorphonuclear leukocytes; smaller, rounded mononuclear cells with sparse cytoplasm were identified as lymphocytes.

Animal weights prior to lesioning and at the conclusion of the study were measured.

Data were compared using unpaired two-way Student's *t*-test, with StatView-2 software. Data are presented as mean ± SEM.

RESULTS

All animals survived the 2-week experimental period. Residual fluid volumes in all pumps were approximately 0.1 cc, indicating appropriate pump function without blockage during the experimental period. Nissl staining revealed accurate intraseptal placement of the infusion catheter in 16 of 17 subjects (Fig. 1). In the 17th subject, a vehicle-infused animal, the infusion catheter tip rested

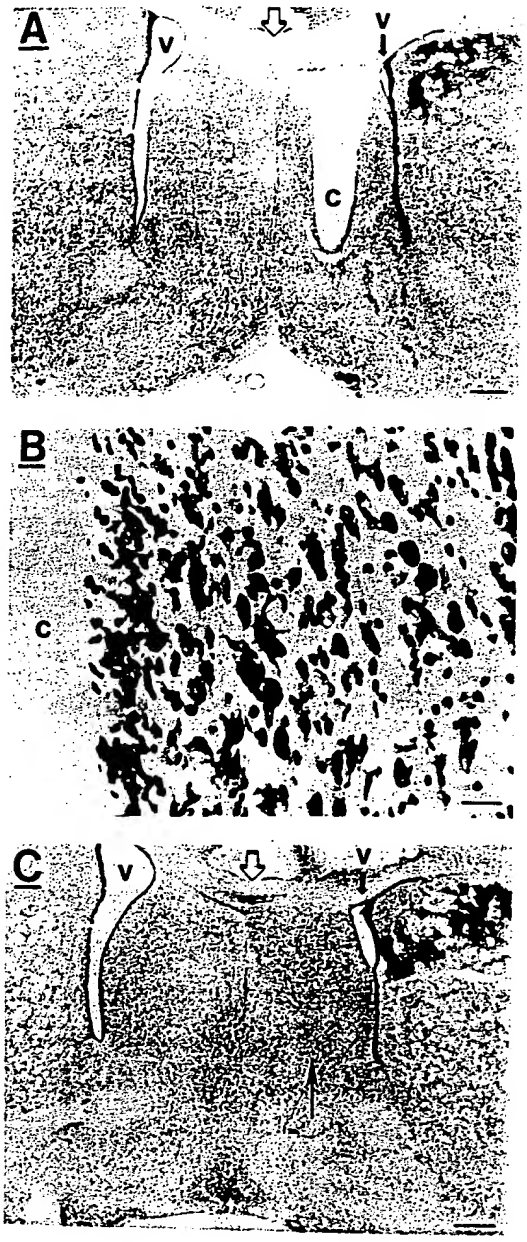

Figure 1. Nissl stain of 2-week intraparenchymal NGF infusion site. No parenchymal damage is evident at sites of NGF infusion. (A) Low-power micrograph of septal intraparenchymal cannulation/infusion site (indicated by "c"). The tract made by the cannula is clearly evident, and there is no host parenchymal destruction adjacent to this site. The lateral ventricle adjacent to the site of infusion is compressed (v, dark arrow). v, ventricle; broad arrow indicates midline. Scale bar = 500 μm. (B) Higher magnification at interface of cannulation site (c) with host brain reveals modest host inflammatory response consisting of polymorphonuclear cells and increased numbers of reactive glia. Normal neurons are also present. Cystic degeneration and tissue liquefaction are not evident. Scale bar = 50 μm. (C) Septal region located 240 μm from

in the lateral ventricle, and this subject was excluded from further consideration.

In no case was extensive parenchymal destruction observed in the region of the infusion catheter tip (Fig. 1). Higher magnification of the infusion zone on Nissl-stained sections revealed a modest cellular inflammatory response consisting of mononuclear and occasional polymorphonuclear cells in the immediate region of the infusion catheter tip. No observable differences were detected between NGF and control subjects with regard to the nature of the modest inflammatory response. Immunolabeling for GFAP revealed only a modest upregulation of astrocytic processes and a moderate hypertrophy of the astrocyte soma in the region of the catheter tip (Fig. 2). Upregulation of GFAP immunoreactivity was restricted to a 1-mm distance from the infusion tract.

ChAT immunolabeling revealed persistent neuronal labeling in 94.7 ± 6.6% of cholinergic somata in recipients of intraparenchymal NGF infusions, compared to only 21.7 ± 2.6% in artificial CSF-infused animals ($p < 0.0001$; Figs. 3 and 4). The degree of cholinergic protection was of identical extent in individual sections quantified over the rostral-to-caudal extent of the medial septal nucleus, indicating that structures located more distantly from the infusion site were protected as fully as sections located adjacent to the intraparenchymal NGF source (Fig. 5). Generally, the distance from the infusion cannula to the most caudal septal section was 1.0–1.5 mm.

p75 low-affinity neurotrophin receptor labeling revealed a marked degree of cholinergic neuritic sprouting directed toward the site of the intraparenchymal infusion catheter in recipients of NGF infusions, but was not evident in animals that received artificial CSF infusions only (Fig. 6). The sprouting in NGF recipients was most robust closest to the infusion site, and was reduced in intensity more distantly from this site. Nonetheless, prominent sprouting was still observable throughout the ipsilateral septum. After ICV NGF infusions, sprouting of cholinergic axons in the ipsilateral septum has been reported toward the subependymal region, where the NGF concentration is highest. In the present study, the pattern of sprouting was redirected toward the intraparenchymal NGF source. Dense p75 immunolabeling of cholinergic axons was also observed in the contralateral septum in recipients of NGF infusions; the intensity of this labeling was greater than that observed in the contralateral septum in vehicle-infused animals. This con-

area depicted in (A) shows no cyst formation or cavitation. Long arrow indicates site of intraparenchymal infusion in more rostrally located section. Compression of lateral ventricle on side of brain ipsilateral to infusion is still evident, but is less extensive. Scale bar = 500 μm.

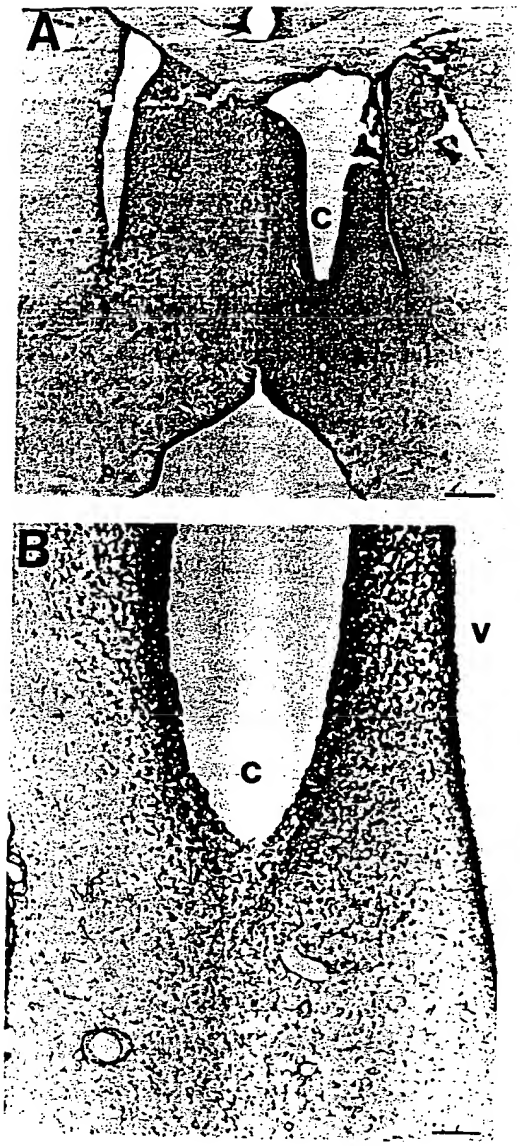

Figure 2. GFAP immunolabel of infusion site. (A) Upregulation of GFAP immunoreactivity in host brain surrounding cannulation site is present, but is modest in extent. Upregulation is primarily restricted to region adjoining cannulation site, and to side of septum ipsilateral to infusion. Tissue tears are an artifactual result of immunolabeling procedure. Scale bar = 380 μm. (B) Higher magnification of GFAP immunoreactivity reveals increased number of GFAP-immunoreactive processes adjacent to cannulation site (c). v, lateral ventricle. Scale bar = 125 μm.

tralateral effect of the NGF infusion could represent either contralateral sprouting of cholinergic axons or NGF-enhanced labeling of cholinergic axons within their normal projection regions. In either case, this enhanced cholinergic labeling represents a contralateral effect of the ipsilateral septal NGF infusion.

Nissl-stained sections through the brain stem did not reveal abnormal proliferation or migration of Schwann cells into the subpial space, whereas this complication has been previously reported after ICV NGF infusions (38). Weight loss was not observed in any experimental subject: artificial CSF-infused subjects gained an average of 2.1 ± 0.3 g over the 2-week experimental time period, and NGF-infused subjects gained an average of 3.1 ± 0.5 g, a difference that was not significant ($p = 0.5$).

DISCUSSION

The present findings indicate that intraparenchymal infusions of NGF can rescue degenerating cholinergic neurons, redirect cholinergic axonal sprouting responses, induce little inflammatory response, and result in no necrosis in host tissue over a 2-week time period. Cholinergic neuronal rescue occurs in equal proportion in serial sections located over the full rostral-to-caudal extent of the medial septal (Ch1) nucleus, despite increasing distance of target cells from the intraparenchymal NGF source. Thus, 2-week delivery of NGF to the region of the cholinergic neuronal soma elicits neuronal rescue without short-term adverse effects. These findings provide a rationale for long-term studies of intraparenchymal infusions of neurotrophic factors in the CNS that would be required to treat such chronic neurodegenerative disorders as Alzheimer's disease.

Previously, Knusel and coworkers reported that intrastriatal injections of NGF induced long-lasting phosphorylation of striatal trk receptors (11,12,34) and increased expression of striatal choline acetyltransferase mRNA (34), indicating that intraparenchymal trophic factor infusion in pharmacological doses is an effective means of eliciting neuronal responses. In the present experiment, NGF was infused intraparenchymally to the septal region, directly adjacent to the region of degenerating cholinergic neurons after fimbria-fornix transections. Nearly complete cholinergic neuronal protection was observed after 2 weeks, and the degree of this protection paralleled that previously described in experiments using similar doses of NGF infused ICV (2,7,14,37) wherein NGF was transported in a specific retrograde fashion (20). Further, surprisingly little parenchymal damage and glial response was observed, potentially supporting the practicality of intraparenchymal infusions as a means of delivering trophic factors to the brain. NGF was infused at a rate of 7.1 μl/day, delivering 0.71 μg NGF/day. Over the 2-week period of this experiment, adverse effects of NGF delivery of the type previously described after intraventricular infusions were not observed, including weight loss or Schwann cell proliferation in the subpial region (18,24,38). Adverse effects of ICV NGF infusions have previously

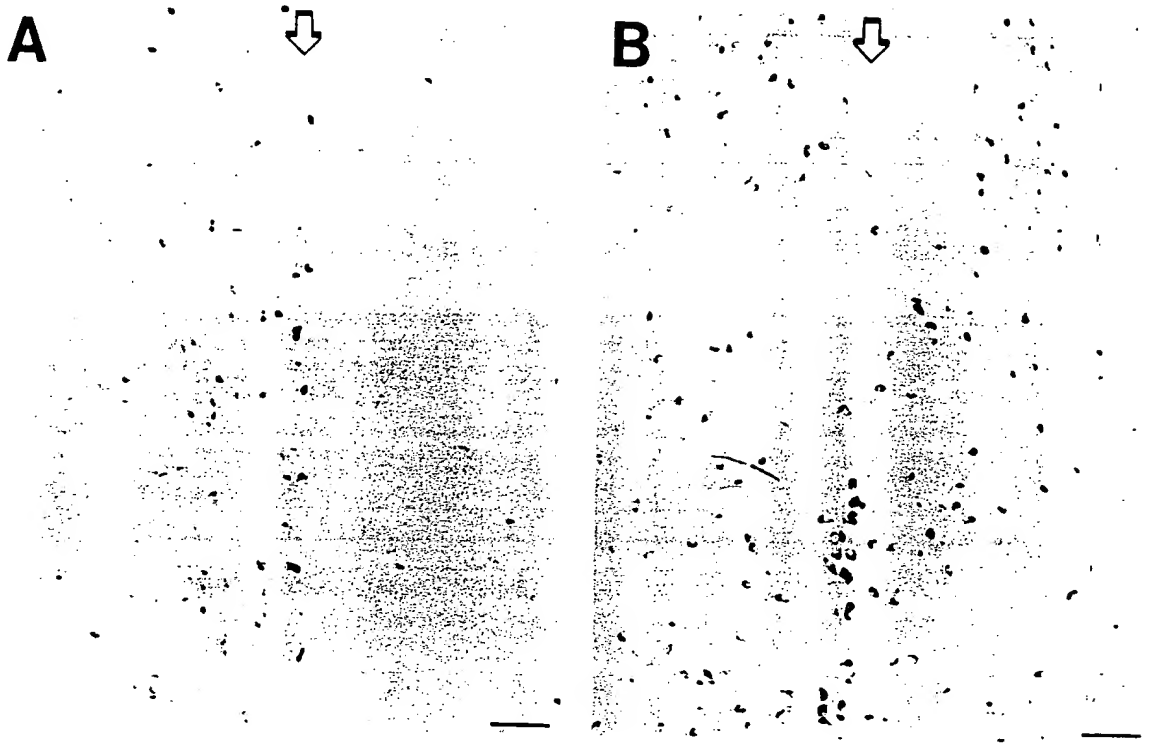

Figure 3. ChAT immunolabel of basal forebrain cholinergic neuronal rescue. (A) In control animal, right-sided fimbria-fornix lesion results in downregulation of ChAT immunoreactivity on side of lesion. Normal numbers of immunolabeled neurons are evident on intact, left side of septal region. (B) Retrograde degeneration of cholinergic neurons is prevented after right-sided fimbria-fornix lesions by intraparenchymal NGF infusions. Arrow indicates midline. Scale bar = 200 μm.

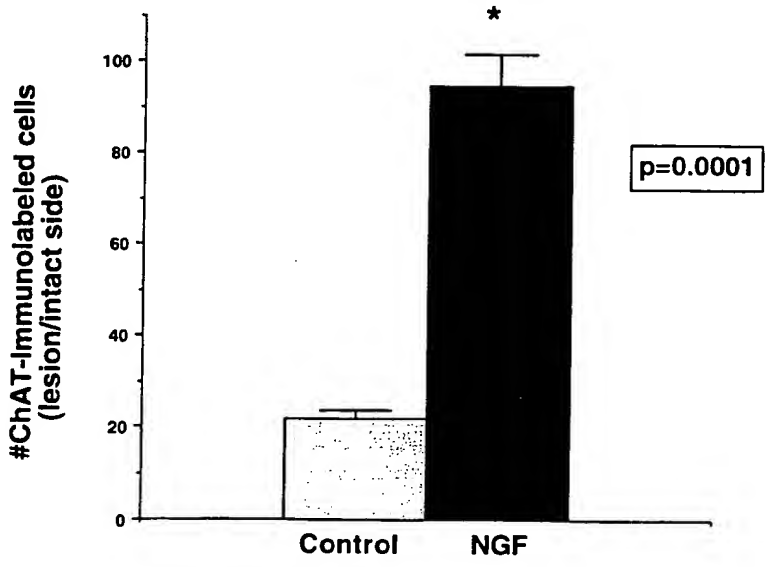

Figure 4. Quantification of basal forebrain cholinergic neuronal rescue. Numbers of ChAT-immunoreactive neurons were measured in three equally spaced sections located 420, 500, and 640 μm rostral to the decussation of the anterior commissure in each animal. Values were expressed as a percentage of neuron numbers compared to the contralateral, intact side of the brain ($n = 7$ NGF-infused and 9 vehicle-infused subjects).

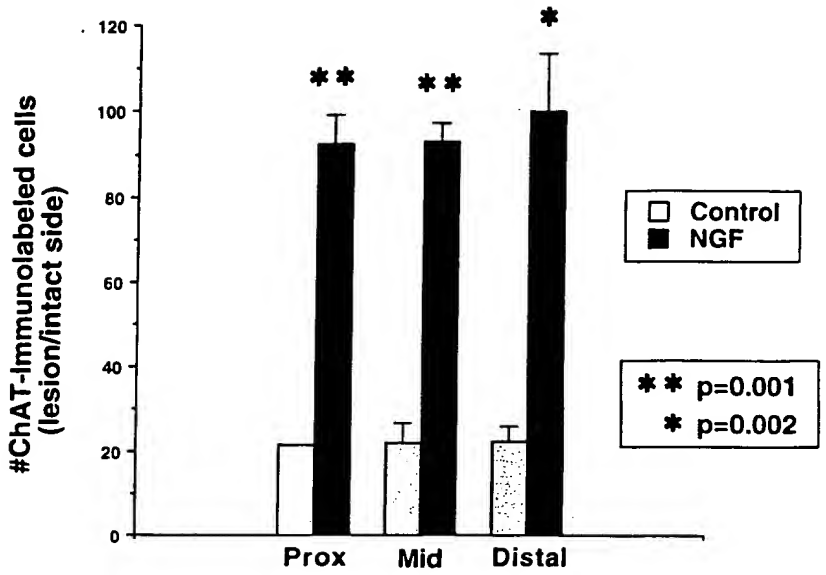

Figure 5. Rostral-to-caudal extent of rescue of basal forebrain cholinergic neurons. The relative proportion of ChAT-immunolabeled neurons as a function of distance from the NGF or vehicle infusion site was quantified. Neuronal rescue after NGF infusion was equally extensive in all sampled sections.

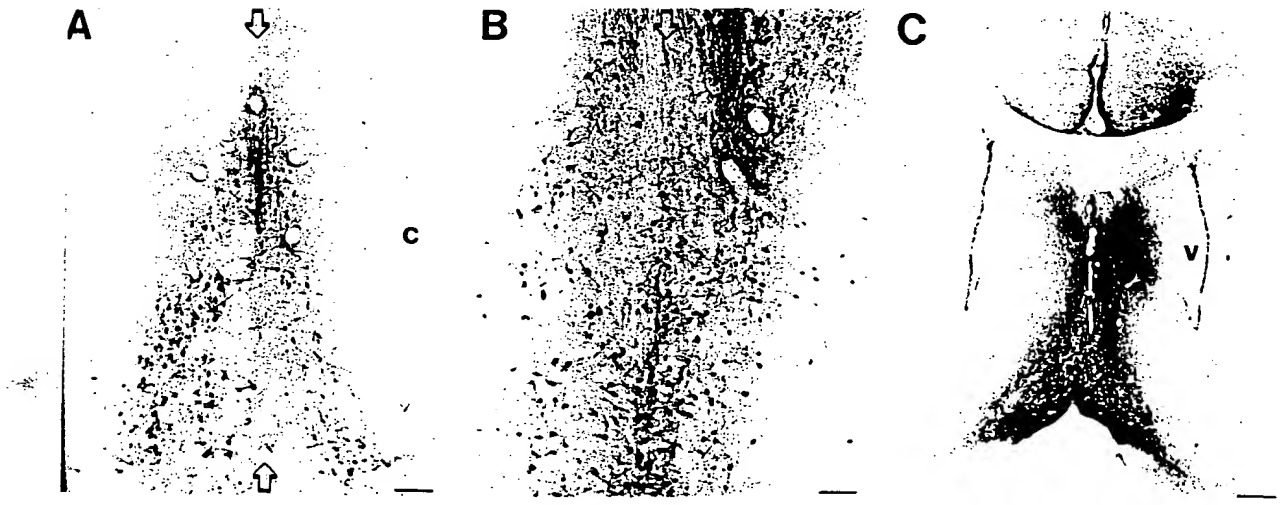

Figure 6. p75 immunolabel of cholinergic neurons and axons. (A) In lesioned control animal, the number of p75-immunolabeled neurons is reduced in the medial septal nucleus ipsilateral to the side of the fimbria-fornix lesion. No sprouting of p75-immunoreactive axons is evident toward the intraparenchymal cannulation site (c) where artificial CSF has been infused. Arrows indicate midline. Scale bar = 312 μm. (B) In subject that received right-sided fimbria-fornix lesion and intraparenchymal NGF infusion, rescue of neuronal immunolabeling and robust sprouting of p75-immunolabeled axons toward the infusion site is evident (dark arrow). Scale bar = 312 μm. (C) Sprouting response toward intraparenchymal NGF infusion site (arrow) is again evident at lower magnification. v, lateral ventricle. Scale bar = 500 μm.

been detected as early as 2 weeks after the start of infusions (Winkler, personal communication), but longer time periods of NGF intraparenchymal delivery must be examined to adequately assess the long-term safety and efficacy of this method. Morse et al. (20) reported a similar degree of protection of the cholinergic phenotype (86%) after intraparenchymal NGF injections in fimbria-fornix-lesioned animals, but did not examine cholinergic protection as a function of distance from the infusion site, axonal sprouting, tissue/glial responses to injection sites, or the development of adverse effects. Also supporting findings of the present study, amelioration of behavioral deficits in rats after a single intraseptal NGF injection has been reported (10), together with an increased size in cholinergic neuronal perikarya in the magnocellular basal forebrain nucleus after intraparenchymal NGF infusions following excitotoxic lesions (5,9). Whether these biological effects of NGF can be attributed solely to activation of somal-based NGF receptors is uncertain; NGF can diffuse for distances of at least 1 cm after intraparenchymal infusions (J. Conner and M. Tuszynski, unpublished observations), thereby potentially activating NGF receptors located on cholinergic axons.

Injured cholinergic axons sprouted toward the intraparenchymal NGF source in the present study, a distinct change from sprouting patterns observed in the direction of the lateral septal subependymal zone after ICV NGF infusions (32,37). Intrahippocampal NGF infusions into adult rats have also been reported to elicit axonal growth responses from transplanted sympathetic axons in an apparent gradient-dependent fashion (4). These altered patterns of axonal sprouting highlight the structural plasticity of neurons in the adult brain, which clearly remain responsive to neuronal growth factors both in trophic (survival) and tropic (directional growth) characteristics. Hippocampal projections to the septal region normally terminate in the lateral septum (28), and ICV NGF infusions might disrupt these projections by recruiting cholinergic axon sprouting into the region. Intraparenchymal NGF infusions shift the topography of cholinergic axonal sprouting away from the lateral septum and could therefore minimize disruption of septal circuitry; however, the functional consequences of increased cholinergic axonal sprouting in the mid and lateral septum resulting from intraparenchymal infusions will require further study with functional assays (21).

In conclusion, a 2-week NGF infusion into a single intraparenchymal site can rescue degenerating cholinergic septal neurons over the full rostral-to-caudal extent of the septal nucleus, without inducing adverse effects associated with ICV NGF infusions. Cholinergic axonal sprouting responses are also redirected, demonstrating the plasticity and responsiveness of injured adult axons to neurotrophic factors. Longer term studies characterizing the distance of NGF diffusion and the rostral/caudal extent of cholinergic protection should be undertaken in the larger brains of nonhuman primates to determine whether intraparenchymal NGF infusion is a clinically practical approach for testing the hypothesis that NGF treatment will reduce cholinergic neuronal degeneration and improve cognition in Alzheimer's disease.

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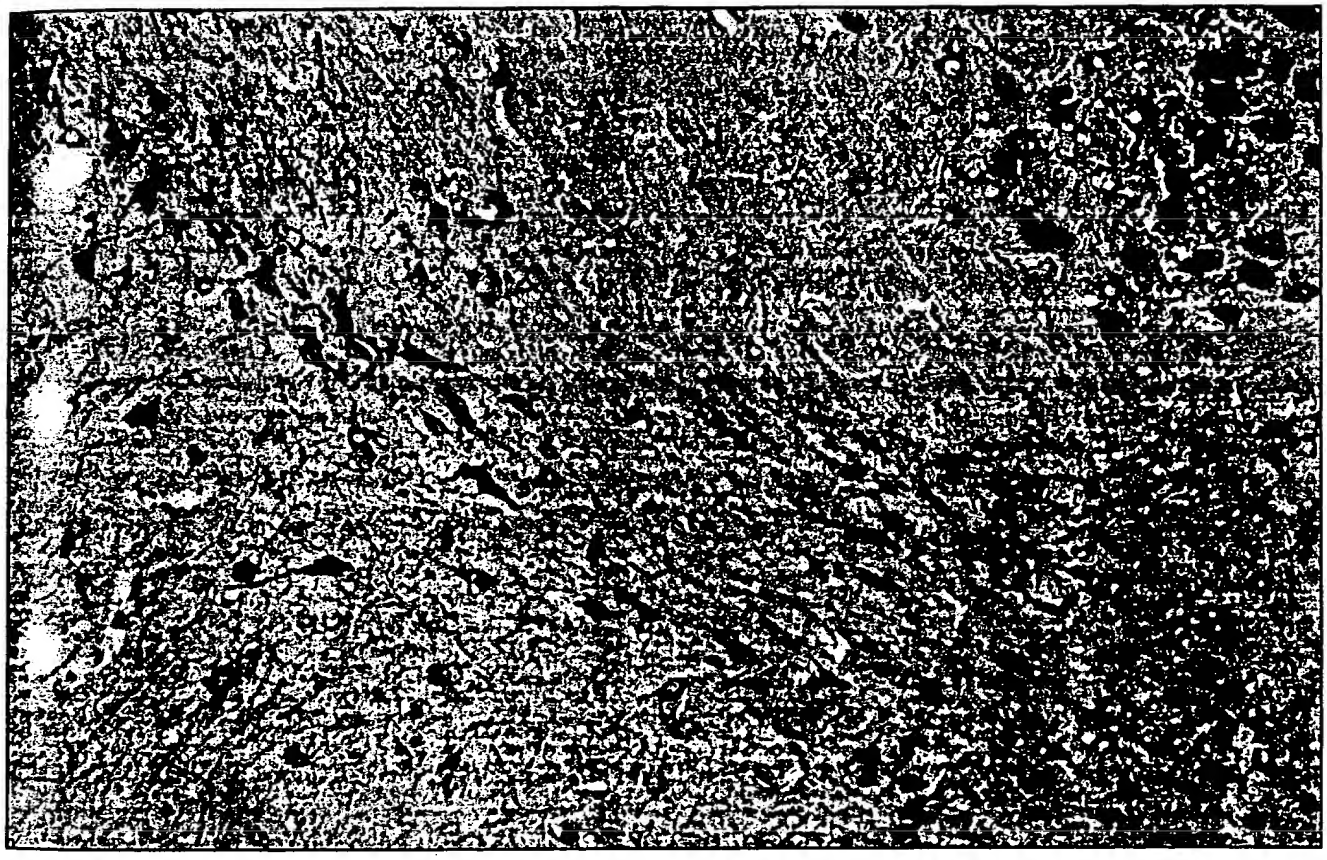
CELL TRANSPLANTATION



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